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Ionic-Liquid-Supported Peptide Synthesis Demonstrated by the Synthesis of Leu^5-enkephalin

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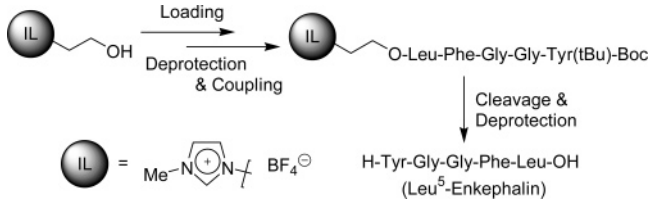

A new strategy for the synthesis of oligopeptides was developed using an ionic liquid as a soluble support. The efficiency of this ionic liquid-phase approach was demonstrated by the synthesis of a bioactive pentapeptide, Leu^5-enkephalin, in good yield and reasonable purity. The structures and purities of the reaction intermediates in each step were verified easily by routine spectroscopic analysis, and no chromatographic procedures were needed during the synthesis.

Introduction

The solid-phase peptide synthesis, introduced by Merrifield in 1963, has been a milestone in the annals of organic synthesis.[1] The facile purification process of removing excess reagents and side products allows for the ease of product isolation and makes automation possible. However, despite the many benefits offered by the solid-phase approach, the heterogeneous nature of the insoluble polymers and reaction conditions often results in a series of problems including nonlinear reaction kinetics, unequal distribution of and/or access to the reaction sites, solvation problems, and a retarded coupling rate. This has led to alternative methodologies with the aim of restoring homogeneous reaction conditions. In recent years, the use of soluble polymer supports has received considerable attention because such a "liquid-phase" synthesis retains many of the advantages of conventional solution chemistry and still permits the fairly easy purification of the product. Soluble polyethylene glycol (PEG), polyvinyl alcohol, and other polymers have been employed successfully in the synthesis of oligopeptides.[2] Some limitations that were expressed for the use of soluble polymer supports include the following: low loading capacity, limited solubility during the synthesis of longer peptides, aqueous solubility, and insolubility in ether solvents.[3] More recently, the use of the "fluorous phase" for organic synthesis has been advocated.[4] This is based on the concept that fluorinated compounds will preferentially dissolve in a fluorous solvent, the fluorous phase. Furthermore, a phase separation can be achieved between a fluorous phase and an organic phase by a temperature switch, thus facilitating separation and purification. So far, the use of fluorous-phase methodology has been demonstrated for the synthesis of oligopeptides,[5] oligosaccharides,[6] and small molecules.[4] Nevertheless, the expense of perfluoroalkane solvents and the need for specialized reagents may limit its general application.

Recently, ionic liquids (ILs) have attracted considerable interest as environmentally benign reaction media because of their many fascinating and intriguing properties such as high thermal and chemical stability, no measurable vapor pressure, nonflammability, friction reduction,

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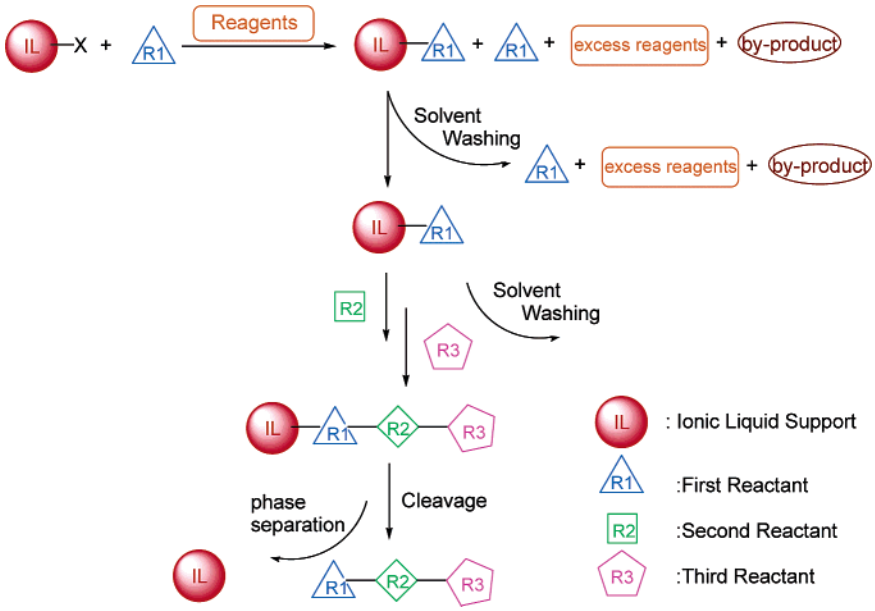
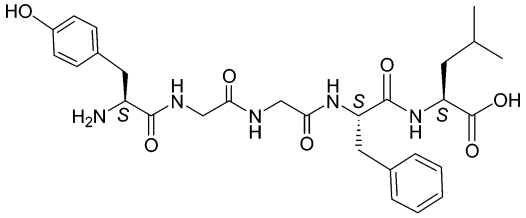


FIGURE 1. General concept of ionic-liquid-supported synthesis.

antiwear performance, and high loading capacity.[7] Numerous chemical reactions, including some enzymatic reactions, can be carried out in ionic liquids.[8] In most cases, the ionic liquids can be recycled easily. An attractive feature of ionic liquids is that their solubilities can be tuned readily, so they can phase separate from organic as well as aqueous media, depending on the choice of cations and anions. Substrate solubility can also be tuned.[9] This suggests the possibility of using these low molecular weight ionic liquids as soluble supports for organic synthesis. Phase separation between the "ionic liquid phase", the less polar organic phase, and the aqueous phase can be achieved for product separation and purification. An illustration of ionic-liquid-supported synthesis (ILSS) is given in Figure 1. After the first reactant is anchored to an ionic liquid support, the excess reagents and byproducts in subsequent reactions can be removed easily by simple solvent washing. Substrates anchored on ionic liquids are expected to retain their reactivities, as in solution reactions, and allow the use of conventional spectroscopic analysis during the synthetic process. Recent reports from our lab and other groups have successfully demonstrated the efficiency of ILSS for small molecules.[10] The possibilities of developing recoverable and recyclable ionic-liquid-supported catalysts have also been explored.[11] Here, we report the application of the ILSS strategy for oligopeptide synthesis[12] using the synthesis of the bioactive pentapeptide Leu^5-enkephalin for demonstration.[13] The issues that we need to address are the following: (1) is the ionic liquid support compatible with the synthetic methodologies generally developed for peptide synthesis? (2) would the presence of the ionic liquid moiety lead to racemization or epimerization of the peptide units during attachment, coupling, and cleavage reactions? and (3) would the oligopeptide chain modify the solubility of the ionic liquid support to the extent that purification via washing with organic and aqueous solvents is no longer meaningful?



Leu^5-enkephalin

Results and Discussion

1. Attachment to the Ionic Liquid and the Test of Racemization. 3-Hydroxyethyl-(1-methylimidazolium)-tetrafluoroborate (**1**), readily available from the reaction of 1-methylimidazole and 2-bromoethanol,[10a,d] was chosen as a suitable ionic liquid support for peptide synthesis. Various coupling reaction conditions (Scheme 1) were examined for the loading of the first amino acid,

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(12) Peptide synthesis using ionic liquids as reaction media has been reported. See Vallette, H.; Ferron, L.; Coquerel, G.; Gaumont, A.-C.; Plaquevent, J.-C. *Tetrahedron Lett.* **2004**, *45*, 1617−1619.

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SCHEME 1

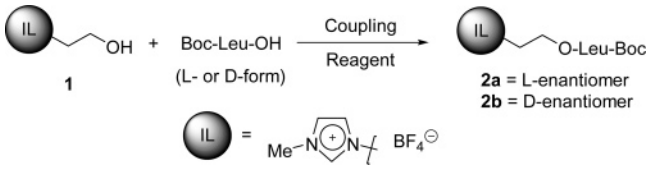


TABLE 1. Loading of the First Amino Acid (Boc-Leu-OH) onto the Ionic-Liquid Support 1

entry	coupling reagent[a]	condition	conversion (%)[b]
1	EDC (2 equiv)/HOBt	CH_3CN/rt/48 h	45
2	HATU (2 equiv)/HOBt	CH_3CN/rt/48 h	53
3	PyBOP (2 equiv)/DIPEA	CH_3CN/rt/48 h	55
4	EEDQ (1.5 equiv)	CH_3CN/35 °C/18 h	71
5	EEDQ (1.5 equiv)	CH_3CN/35 °C/72 h	77
6	EEDQ (5 equiv)	CH_3CN/60 °C/18 h	86
7	IIDQ (2 equiv)	CH_3CN/35 °C/48 h	87
8	DCC (2 equiv)/DMAP	CH_3CN/rt/18 h	100

[a] Equivalent of the coupling reagent to Boc-Leu-OH. [b] Determined by ^1H NMR analysis.

Boc-leucine, onto **1**, and the results are shown in Table 1. Among the conditions we have tried, the dicyclohexylcarbodiimide (DCC)/4-(*N*,*N*-dimethylamino)pyridine (DMAP) combination gave the best conversion. Other methods including 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC)/1-hydroxylbenzotriazole hydrate (HOBt), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)/HOBt, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)/diisopropylethylamine (DIPEA), 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), and 2-isobutoxy-1-isobutoxycarbonyl-1,2-dihycroquinoline (IIDQ) were not able to push the esterification reaction to quantitative conversion. Although capping of unreacted **1** with acetic anhydride worked well and could be used as an alternative, we have settled on using the DCC/DMAP conditions for the coupling reaction and avoided the need for the capping step. The excess Boc-leucine, the DCU byproduct, and DMAP could all be removed by sequential ether and aqueous-acid washings. The ionic-liquid-supported Boc-leucine (**2**) was obtained in high yield (91%) and good purity, as verified by NMR spectroscopy. We have coupled both L- and D-Leu-Boc to give **2a** and **2b**, respectively.

The deprotection of the Boc group in **2a** was then studied (Table 3). Both tetrafluoroboric acid and trifluoroacetic acid could induce a very clean deprotection reaction, as indicated by proton NMR. We have also tried to accomplish the deprotection reaction with acidic ion-exchange resins such as Dowex 50Wx8-100 or Amberlyst 15, but the results were not satisfactory. For the next step, the coupling with Boc-Phe-OH, did not work well without neutralization (Scheme 2 and Table 2). Attempts to neutralize TFA or HBF_4 with $NaHCO_3$, Et_3N, and basic ion-exchange resins, such as Dowex 550A OH resin, still could not get the coupling reaction to proceed in high yield. Eventually, we were able to find that using the PyBOP/DIPEA conditions both the neutralization and the coupling reaction happened in one pot. The peptide formation reaction gave **4a** in essentially quantitative yield.[14] It is noteworthy that the use of a large excess of reagents was not necessary. We have tested the reaction with only 1.2 equiv of Boc-Phe-OH and PyBOP, and the result was still satisfactory.

TABLE 2. Deprotection and Coupling of Ionic-Liquid Support Leu-Boc 2a with Boc-Phe-OH

entry	deprotection/ neutralization	coupling condition[a]	conversion (%)[b]
1	TFA/no neutralization	EEDQ (2.5 equiv)/CH_3CN/ 35 °C/48 h	55
2	TFA/no neutralization	DCC (2 equiv)/DMAP/CH_3CN/ rt/48 h	60
3	TFA/$NaHCO_3$	EEDQ (2.5 equiv) /CH_3CN/ 35 °C/48 h	60
4	TFA/Et_3N	IIDQ (2 equiv) /CH_3CN/35 °C/ 48 h	85
5	TFA/$NaHCO_3$	DCC (2 equiv)/DMAP/CH_3CN/ rt/48 h	85
6	TFA/Et_3N	DCC (2 equiv)/DMAP/CH_3CN/ rt/48 h	95
7	TFA/DIPEA	PyBOP (1.5 equiv)/DIPEA (3 equiv)/ CH_3CN/35 °C/8 h	100
8	HBF_4/OH^- resin	EEDQ (2.5 equiv)/CH_3CN/ 35 °C/48 h	25
9	HBF_4/$NaHCO_3$	EEDQ (2.5 equiv)/CH_3CN/ 35 °C/48 h	60

[a] Equivalent of the coupling reagent to Boc-Phe-OH. [b] Determined by ^1H NMR analysis.

TABLE 3. Comparison of Conventional and Various Supported Peptide-Synthesis Methods[a]

aspect	CSPPS	SPPS	SPSPS	FPPS	ILSPS
generic protocol	−	+	+	+	+
homogeneous synthesis	+	−	+	+	+
high loading capacity	n/a	−	−	+	+
low excess reagents	+	−	+	+	+
facile purification	−	+	+	+	+
routine spectroscopic analysis	+	−	±	+	+
relatively low cost	±	−	−	−	+

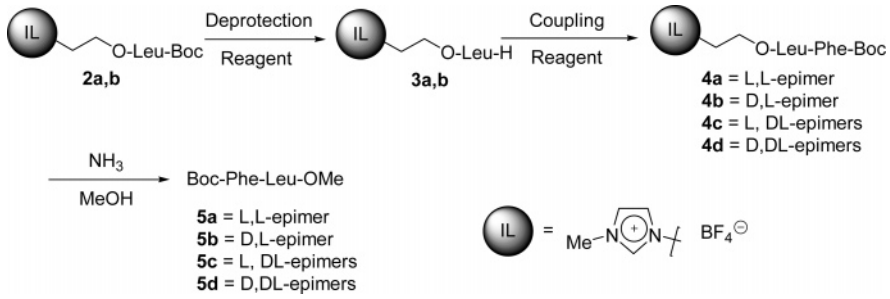
[a] CSPPS: conventional solution-phase peptide synthesis; SPPS: solid-phase peptide synthesis; SPSPS: soluble-polymer-supported peptide synthesis; FPPS: fluorous-phase peptide synthesis; ILSPS: ionic-liquid-supported peptide synthesis.

To determine the degree of the possible racemization or epimerization that may occur during the ILSPS process, especially in the first step of esterification (loading) using the DCC/DMAP condition, we synthesized a series of ionic-liquid-supported dipeptides (**4a**−**d**) and cleaved them with ammonia/methanol to give dipeptides **5a**−**d**, which contain all of the four stereoisomers of Boc-Phe-Leu-OMe (Scheme 2). Careful HPLC analysis showed that no detectable amounts of other isomers (<0.5%) were present in the **5a**-(L, L) or **5b**-(D, L) samples thus prepared, indicating that in both of the loading and peptide forming steps in ILSPS synthesis no significant racemization or epimerization occurred. Coincidentally, this also suggests that no epimerization occurred during the cleavage step under basic methanolic conditions.

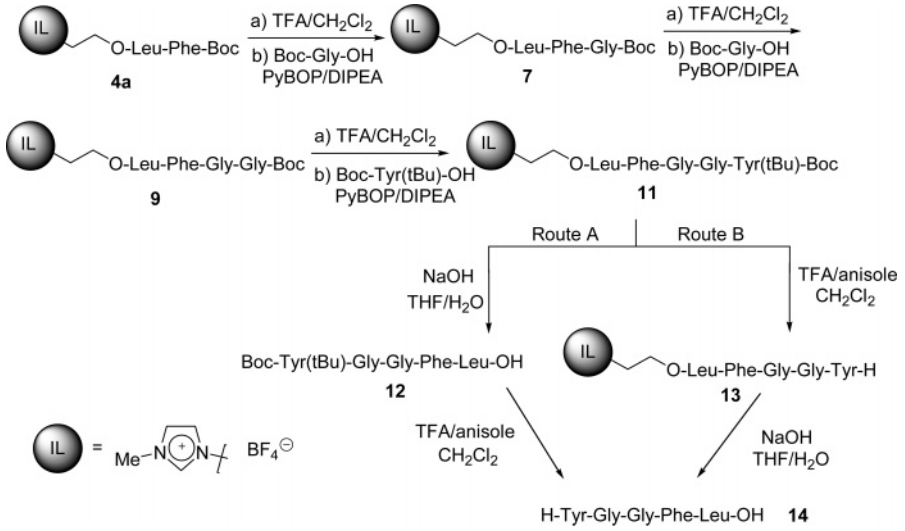
2. Oligopeptide Synthesis and Detachment from the Ionic Liquid. Utilizing the above conditions that were developed for deprotection and coupling, two glycine moieties and one tyrosine moiety in their protected forms were successively coupled to **4a** to give the ionic-liquid-supported pentapeptide (**11**) as a foamlike pale yellow solid (Scheme 3). All of the ionic-liquid-supported peptides prepared thus far are soluble in polar organic solvents such as acetone, acetonitrile, methanol, chloroform, and dichloromethane but are essentially not soluble in diethyl ether or hexane. During the whole synthetic

(14) For a recent monograph on peptide synthesis, see Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997.

SCHEME 2



SCHEME 3



sequence, all of the intermediates in deprotected form (**6**, **8**, and **10**) or Boc protected form (**7**, **9**, and **11**) were isolated and purified by the generic protocol of sequential organic solvents and aqueous washing (see the Experimental Section). Structural confirmation and purity analysis were realized easily by conventional methods such as NMR (acetone-d_6) and MS. The presence or absence of the *tert*-butyl protons of the Boc group in the ^{1}H NMR spectrum was characteristic of the coupling or deprotection step, respectively. The mass spectra of the ionic-liquid-supported oligopeptides (**2a**, **4a**, **7**, **9**, and **11**) were also helpful for the structural characterization because the peak corresponding to the cation bearing the oligopeptide was detected easily as the most intense peak in the spectrum (see the Supporting Information).

Two routes were tested for liberating the final pentapeptide product, Leu^5-enkaphalin **14**, from the ionic liquid support. Route A was to cleave the ester linker bond under basic aqueous conditions first and then deprotect the Boc and tBu groups under TFA/anisole conditions. Route B was to perform the deprotection first and then the cleavage (Scheme 3). Both were successful, but route A was chosen as the preferred procedure for releasing the peptide from the ionic-liquid support in 84% yield because the protected pentapeptide (**12**) could precipitate out easily from the reaction system upon acidification in good yield and purity.

We now had to determine the purity of the Leu^5-enkephalin pentapeptide (**14**) thus prepared. Compound **14**, obtained in 50% overall yield from **1** *without any recrystallization or chromatography procedure* as the TFA salt, was found to have physical properties {mp 148−152 °C and $[\alpha]^{20}_D = 25.0$ (c 0.8, 95% AcOH)} nearly the same as an authentic sample {[mp 150−153 °C and $[\alpha]^{20}_D = 25.6$ (c 0.9, 95% AcOH)].[15] Its identity was further confirmed by its ^{1}H NMR spectrum, which was identical to that of the authentic sample (Figure 2). Its purity was

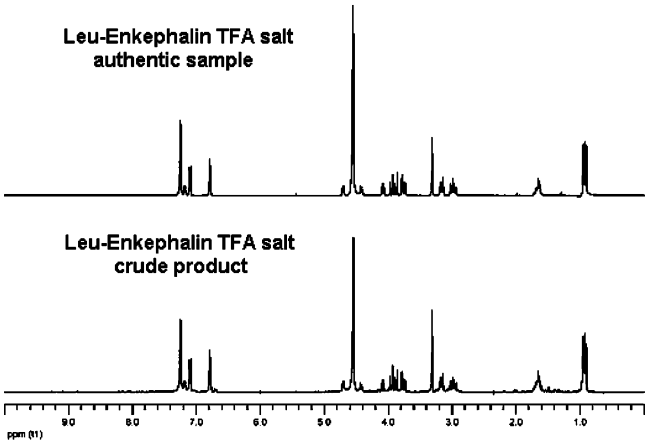


FIGURE 2. ^{1}H NMR spectra of authentic and synthetic Leu^5-enkephalin **14**.

inferior to the authentic sample (>97%) and demonstrated by HPLC analysis (Figure 3) to be about >90% pure. Such a level of purity is superior to what could usually be obtained by solid-phase peptide synthesis prior to chromatography purification.[14]

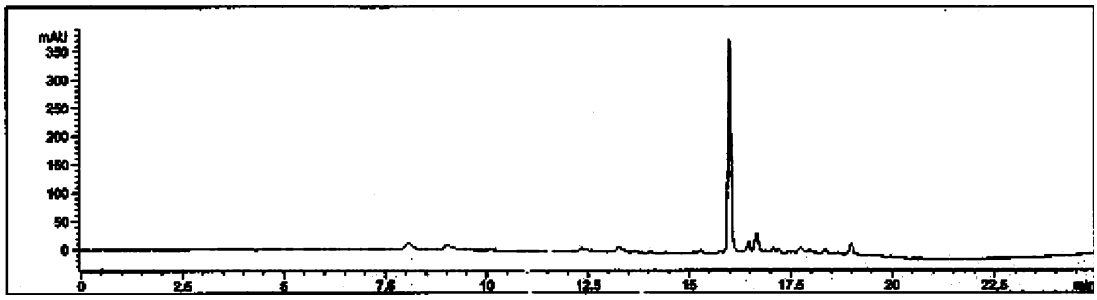


FIGURE 3. HPLC analysis of crude Leu[5]-enkephalin **14** synthesized by ILSPS.[16]

3. Pros and Cons of Ionic-Liquid-Supported Peptide Synthesis (ILSPS). In conclusion, a novel solution-phase approach to oligopeptide synthesis has been developed based on the ionic-liquid-support strategy with a generic protocol of coupling and purification. On the basis of this example, the ILSPS approach offers some potential advantages as well as limitations in comparison to the existing methods of peptide synthesis (Table 3). First, in common with other solution phase methodologies, the use of a large excess of reaction reagents can be avoided in contrast to the solid-phase peptide synthesis (SPPS). This will be important for the synthesis of peptides containing unnatural amino acids (e. g., D-amino acids) or large scale synthesis. Second, in each step, the intermediate was purified easily by solvent washings. In this respect, the ILSPS is similar to the fluorous-phase peptide synthesis (FPPS) and the soluble-polymer-supported peptide synthesis (SPSPS) in using liquid/liquid phase separation. The separation is probably not as easily automated as the SPPS, which uses solid/liquid phase separation. However, the loading capacity of ILSPS is higher because only a molar equivalent of the low molecular weight ionic liquid (**1**) is used. Furthermore, the cost of **1** is probably lower than the fluorous support or even the polymer support, and this may be an important consideration for large scale synthesis. Another advantage of ILSPS is that the structure and purity of each intermediate in the synthesis could be verified easily by routine spectroscopic methods. However, the ILSPS has so far been applied to a relatively short pentapeptide. Whether the methodology is applicable to longer oligopeptides remains to be determined, especially if the longer peptide chain may modify the solubility characteristics of the ionic liquid moiety. However, it is clear that the ILSPS offers potential advantages, and further research into the scope of ILSPS is warranted.

Experimental Section

Esterification (Loading) Step. Dicyclohexylcarbodiimide (DCC, 1 M in CH_2Cl_2, 10 mL, 10 mmol) was added to a mixture of ionic liquid **1** (1.07 g, 5 mmol), Boc-leucine (2.31 g, 10 mmol), and (dimethylamino)pyridine (0.25 g, 2 mmol) in dry acetonitrile (25 mL). The mixture was stirred vigorously for 18 h at room temperature under nitrogen and filtrated through a plug of Celite. The Celite plug was rinsed with acetonitrile, and the combined organic phase was concentrated in vacuum. The crude residue was washed first with ether (20 mLx3) and then dissolved in CH_2Cl_2 and washed with 2 M HCl (10 mLx3). The organic phase was dried over Na_2SO_4 and concentrated to afford 1.95 g (91%) of ionic-liquid-supported Boc-leucine **2a** as a pale yellow oil.

Peptide-Forming Step (Deprotection and Coupling). TFA (10 mL) was added to a solution of ionic-liquid-supported Boc-peptide (2 mmol) in CH_2Cl_2 (10 mL). The reaction was stirred at room temperature under nitrogen for 0.5 h. Upon concentration under reduced pressure, the residue was washed twice with ether and dried on a vacuum line to yield the deprotected ionic-liquid-supported peptide as a TFA salt. The residue was then dissolved together with the next Boc-protected amino acid (3 mmol) and PyBOP (3 mmol) in CH_3CN (35 mL). DIPEA (6 mmol) was added dropwise, and the resulting reaction mixture was stirred at 35 °C under nitrogen atmosphere for 8 h. The solvent was removed under vacuum, and the residue was washed first with ether (20 mL × 3) and then dissolved in CH_2Cl_2 and washed with water (10 mL × 3). The organic phase was dried over Na_2SO_4 and concentrated to afford the ionic-liquid-supported Boc-peptide (90% yield), which was of sufficient purity and could be used directly for the next cycle of peptide synthesis. The product could be purified further, if desired, by dissolving the compound in acetone, filtering through a short pad of silica gel, and evaporating the solvent.

Peptide-Liberation Step. NaOH aqueous solution (1 M, 0.2 mL) was added to a mixture of the ionic-liquid-supported pentapeptide (**11**, 180 mg, 0.2 mmol) in THF/H_2O (1:2, v/v, 3.0 mL). The mixture was stirred at room temperature for 5 h. The volatile component was removed under reduced pressure, and the residue solution was acidified to pH 5−6. The precipitate was washed twice with distilled water and dried on a vacuum line to give 120 mg (85%) of Boc-pentapeptide **12** as a foamlike pale-yellow solid. This oligopeptide (117 mg, 0.16 mmol) was dissolved in CH_2Cl_2 (1.5 mL) and then TFA (1.5 mL), and several drops of anisole were added. The mixture was stirred at ambient temperature under nitrogen atmosphere for 0.5 h. Evaporation and washing with ether gave Leu[5]-enkephalin TFA salt **14** (109 mg, 99%).

Acknowledgment. We thank Merck Frosst Canada and the Natural Science and Engineering Research Council (NSERC) of Canada for financial support.

Supporting Information Available: Experimental details, characterization data of compounds **2**−**12** and **14**, ^1H NMR and mass spectra of ionic-liquid-supported peptides **2**, **4**, **7**, **9** and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) The authentic sample of Leu[5]-enkephalin was purchased from Sigma-Aldrich Canada Ltd. and was reported to be 97% pure.

(16) HPLC conditions: column, Agilent Zorbax SB-CN Semi-prep (9.4 × 250 mm); eluent, 0−5 min, H_2O/0.05% TFA (v/v), 6−15 min, 0−60% CH_3CN/H_2O/0.05% TFA (v/v/v), 16−23 min, 60−100% CH_3CN/H_2O/0.05% TFA (v/v/v); post time, 7min; flow rate, 3.0 mL/min.